

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 49/00		A1	(11) International Publication Number: WO 00/50093 (43) International Publication Date: 31 August 2000 (31.08.00)
(21) International Application Number: PCT/US00/01322		(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 20 January 2000 (20.01.00)		Published <i>With international search report.</i>	
(30) Priority Data: 09/258,148 26 February 1999 (26.02.99) US			
(71) Applicant: MALLINCKRODT INC. [US/US]; 675 McDonnell Boulevard, P.O. Box 5840, St. Louis, MO 63134 (US).			
(72) Inventors: DORSHOW, Richard, Bradley; 11977 Nichlaus Lane, St. Louis, MO 63146 (US); ACHILEFU, Samuel; 3424 San Sevilla Court, St. Louis, MO 63044 (US); RAJAGOPALAN, Raghavan; 13031 Vinson Court, Maryland Heights, MO 63043 (US); BUGAI, Joseph, Edward; 2916 Kettering Drive, St. Charles, MO 63303 (US).			
(74) Agents: PEPPER, George, R. et al; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street, N.W., Columbia Square, Washington, DC 20004 (US).			
(54) Title: METHOD OF MEASURING PHYSIOLOGICAL FUNCTION			
(57) Abstract			
A method of measuring physiological function of a group of body cells, includes the step of selecting a detectable agent capable of emitting a measurable electromagnetic emission. The agent is introduced into body fluid which contacts the group of body cells. The emission is measured, and physiological function is determined based on measurement of the emission.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finnland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxemburg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Iceland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 49/00		(11) International Publication Number: WO 00/50093 (43) International Publication Date: 31 August 2000 (31.08.00)
<p>(21) International Application Number: PCT/US00/01322</p> <p>(22) International Filing Date: 20 January 2000 (20.01.00)</p> <p>(30) Priority Data: 09/258,148 26 February 1999 (26.02.99) US</p> <p>(71) Applicant: MALLINCKRODT INC. [US/US]; 675 McDonnell Boulevard, P.O. Box 5840, St. Louis, MO 63134 (US).</p> <p>(72) Inventors: DORSHOW, Richard, Bradley; 11977 Niehaus Lane, St. Louis, MO 63146 (US). ACHILEFU, Samuel; 3424 San Sevilla Court, St. Louis, MO 63044 (US). RAJAGOPALAN, Raghavan; 13031 Vinson Court, Maryland Heights, MO 63043 (US). BUGAJ, Joseph, Edward; 2916 Kettering Drive, St. Charles, MO 63303 (US).</p> <p>(74) Agents: REPPER, George, R. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, 555 13th Street, N.W., Columbia Square, Washington, DC 20004 (US).</p>		<p>(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report.</i></p>
<p>(54) Title: METHOD OF MEASURING PHYSIOLOGICAL FUNCTION</p> <p>(57) Abstract</p> <p>A method of measuring physiological function of a group of body cells, includes the step of selecting a detectable agent capable of emitting a measurable electromagnetic emission. The agent is introduced into body fluid which contacts the group of body cells. The emission is measured, and physiological function is determined based on measurement of the emission.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	NB	Niger	US	United States of America
CA	Canada	IT	Italy	NL	Netherlands	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NO	Norway	VN	Viet Nam
CG	Congo	KE	Kenya	NZ	New Zealand	YU	Yugoslavia
CH	Switzerland	KG	Kirgyzstan	PL	Poland	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PT	Portugal		
CM	Cameroon	KR	Republic of Korea	RO	Romania		
CN	China	KZ	Kazakhstan	RU	Russian Federation		
CU	Cuba	LC	Saint Lucia	SD	Sudan		
CZ	Czech Republic	LI	Liechtenstein	SE	Sweden		
DE	Germany	LK	Sri Lanka	SG	Singapore		
DK	Denmark	LR	Liberia				
EE	Estonia						

METHOD OF MEASURING PHYSIOLOGICAL FUNCTIONBACKGROUND OF THE INVENTIONField of the Invention

The present invention is in the field of measuring
5 physiological function of a group of body cells.

Description of the Background Art

Current clinical practice for determining liver function includes deriving a CTC score, which is a compilation of laboratory data and clinical assessment of ascites and
10 encephalopathy. D.A. Noe and R.C. Rock (eds), Laboratory Medicine, The Selection and Interpretation of Clinical Laboratory Studies, Williams and Wilkins, 1994, Baltimore, MD, Chapter 5, Assessment of Organ Function, by D.A. Noe, p. 55-60, Chapter 19, Liver and Biliary Tract, by A.T. Blei, p. 363-15 379, Chapter 21, The Kidneys, by D.A. Oken and A.C. Schoolwerth, p. 401-410.

Another test involves the use of indocyanine green (ICG). ICG is known to be exclusively cleared from the bloodstream by the liver. Thus a measurement of the ICG blood
20 clearance time profile is directly related to liver function. J. Caesar, S. Shaldon, L. Chiandussi, L. Guevara, and Sheila Sherlock, "The use of indocyanine green in the measurement of hepatic blood flow and as a test of hepatic function," Clin. Sci. 21, 43-57 (1961).

25 The ICG test has undergone an evolution in technology. In its first incarnation, blood was withdrawn from the subject at several times following an IV bolus injection. The blood

samples were then processed spectrophotometrically to determine ICG concentration. R. Jalan and P. C. Hayes, "Review article: quantitative tests of liver function," *Aliment Pharmacol. Ther.* 9, 263-270 (1995); A. W. Hemming, C. 5 H. Scudamore, C. R. Shackleton, M. Pudek, and S. R. Erb, "Indocyanine green clearance as a predictor of successful hepatic resection in cirrhotic patients," *Am. J. Surg.* 163, 515-518 (1992); P. Ott, S. Keiding, and L. Bass, "Plasma elimination of indocyanine green in the intact pig after bolus 10 injection and during constant infusion: comparison of spectrophotometry and high-pressure liquid chromatography for concentration analysis," *Hepatology* 18, 1504-1515 (1993). Subsequently, a non-invasive technique employing ear densitometry was developed. C. M. Leevy, F. Smith, J. 15 Longueville, G. Paumgartner, and M. M. Howard, "Indocyanine green clearance as a test for hepatic function: Evaluation by dichromatic ear densitometry," *Journal of Medicine* 24, 10-27 (1993). Problems associated with the clinical development of this device recently led Japanese researchers to improve upon 20 the ear densitometry technique. This newer method, termed the finger-piece method, employs transmitted light of two wavelengths measured throughout a finger to deduce ICG concentration. M. Kanda, K. Taniguchi, K. Awazu, Y. Ishigami, M. Masuzawa, and H. Abe, "Continuous monitoring of Cardiogreen 25 removal by a diseased liver using an optical sensor," *Proc. SPIE* 904, 39-46 (1988); M. Nakayama, N. Kanaya, S. Fujita, and

A. Namiki, "Effects of ephedrine on indocyanine green clearance during spinal anesthesia: Evaluation by the finger piece method," *Anesth. Analg.* **77**, 947-949 (1993); N. Kanaya, H. Iwasaki, and A. Namiki, "Noninvasive ICG clearance test for estimating hepatic blood flow during halothane and isoflurane anaesthesia," *Can. J. Anaesth.* **42**, 209-212 (1995); N. Kanaya, M. Nakayama, S. Fujita, and A. Namiki, "Comparison of the effects of sevoflurane, isoflurane and halothane on indocyanine green clearance," *Br. J. Anaesth.* **74**, 164-167 (1995); S. Shimizu, W. Kamiike, N. Hatanaka, Y. Yoshida, K. Tagawa, M. Miyata, and H. Matsuda, "New method for measuring ICG R_{max} with a clearance meter," *World J. Surg.* **19**, 113-118 (1995).

Both ear densitometry and the finger-piece method involve measuring absorption (or transmission) of light by ICG.

Also of interest is that in vitro fluorometric determination of ICG in plasma has been demonstrated, B. Hollins, B. Noe, and J.M. Henderson, "Fluorometric determination of indocyanine green in plasma," *Clin. Chem.* **33**, 765-768 (1987).

Other references of general interest include: R.L. Sheridan, et al., "Burn depth estimation by indocyanine green fluorescence: Initial human trial," *Journal of Burn Care & Rehabilitation* **16**, 602-604 (1995); M.A. O'Leary, D.A. Boas, B. Chance, and A.G. Yodh, "Reradiation and imaging of diffuse photon density waves using fluorescent inhomogeneities,"

Journal of Luminescence 60 & 61, 281-286 (1994); X. Li, B. Beauvoit, R. White, S. Nicka, B. Chance, and A. Yodh, "Tumor localization using fluorescence of indocyanine green (ICG) in rat models," Proc. SPIE 2389, 789-797 (1995).

5 There remains a need in the art for improved methods of measuring physiological function.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method of measuring physiological function of a group of body cells, 10 includes the step of selecting a detectable agent capable of emitting a measurable member comprising an electromagnetic emission. The agent is introduced into body fluid which contacts the group of body cells. The emission is measured, and physiological function is determined based on measurement 15 of the emission. The emission can be measured using non-invasive or invasive techniques. Invasive techniques include using endoscopes and catheters inserted into the respective body portion. Non-invasive techniques include surface probes such as ear clips and finger probes.

20 BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of an *in vivo* fluorescence detection apparatus in accordance with one embodiment.

FIG. 2 graphically depicts the *in vivo* fluorescence time 25 dependence after a bolus injection of FITC labelled succinylated poly-D-Lysine in a single rat pre-kidney ligation

(Normal) and post-kidney ligation (Ligated). The solid line is a single exponential fit to the measured data. (Excitation at 488 nm, emission monitored at 518 nm.)

FIG. 3 graphically depicts *in vivo* fluorescence time
5 dependence for three rats after a bolus injection of ICG. The
solid lines are single exponential fits to the measured data.

FIG. 4 graphically depicts the *in vivo* fluorescence time
dependence of a succession of bolus injections in one rat. In
chronological order: ICG (ICG-1), FITC only, saline only, ICG
10 again (ICG-2).

FIG. 5 graphically depicts the *in vivo* fluorescence time
dependence after a bolus injection of ICG in a single rat pre-
partial liver ablation (Normal) and post-partial liver
ablation (Ablated). The solid lines are single exponential
15 fits to the measured data.

FIG. 6 shows the blood clearance profile of fluorescein-
polyaspartic acid (6000) conjugate.

FIG. 7 shows the blood clearance profile of fluorescein-
polyaspartic acid (10000) conjugate.

20 FIG. 8 shows the blood clearance profile of fluorescein-
polyglutamic acid (13000) conjugate.

FIG. 9 shows the blood clearance profile of fluorescein-
polyarginine (10000) conjugate.

25 FIG. 10 shows the blood clearance profile of indocyanine-
mono(polyaspartic acid 2000) and indocyanine-bis(polyaspartic
acid 2000).

FIG. 11 shows the blood clearance profile of indocyanine (polyaspartic acid 6000).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one embodiment of the present invention, a method is disclosed for determining cell and/or organ function by measuring the blood pool clearance of a targeted agent, sometimes referred to herein as tracer. The cell and/or organ function can be determined by the rate these cells remove the tracer from the bloodstream. Function can also be assessed by measuring the rate the cells of interest accumulate the tracer or convert it into an active or other form.

The agent may be targeted to a group of cells or organ which is a high capacity clearance system. The agent may contain a chromophore and/or fluorophore.

For agents containing chromophores and/or fluorophores, blood pool clearance may be measured using a light source/photocell device that measures tissue absorbance or fluorescence in a non-target site, such as an ear lobe, finger, brain or retina. Accumulation of the tracer within the cells of interest can be assessed in a similar fashion. The detection of such accumulation can be facilitated by using fluorophores which emit in the near infrared wavelengths since body tissues are relatively transparent at these wavelengths.

The agent may be introduced into the patient by any suitable method, including intravenous, intraperitoneal or

subcutaneous injection or infusion, oral administration, transdermal absorption through the skin, or by inhalation.

The present invention may be used for rapid bedside evaluation of biologic functions. For example, data on 5 cardiac output, cause of hypercholesterolemia, as well as renal and hepatic function, may be obtained in less than sixty minutes at the bedside after a single intravenous injection. In accordance with one embodiment, a patient may receive a bolus injection of a plurality (e.g., three) different 10 compounds, each containing a different agent (e.g., fluorophore).

Cardiac output may be determined utilizing the present invention in conjunction with known methods such as the Fick principle.

15 Glomerular filtration may be determined by clearance of a low molecular weight fluorescent agent such as fluorescein-succinylated poly-D-Lysine or fluorescein-inulin.

Whether hypercholesterolemia is caused by poor LDL clearance may be determined by analyzing the clearance of 20 fluorescent-labelled LDL. Hepatic function may be assessed by measuring the clearance rate of a fluorescent-labelled asialoglycoprotein or a dye such as indocyanine green.

The present invention includes fluorescence detection of an agent which is cleared from the bloodstream by the kidneys 25 or liver. Thus, assessment of renal or hepatic function by *in vivo* fluorescence detection is encompassed within the

invention. The invention can also be used to monitor the efficiency of hemodialysis.

Tumor cells or brain cells also can be targeted in accordance with the invention.

5 The clearance of a plurality of separate tracers may be determined simultaneously by selecting excitation wavelengths and filters for the emitted photons. The concentration/time curves may be analyzed in real time by a microprocessor. The resulting clearance rates may be calculated and displayed for
10 immediate clinical impact. In cases where unlabeled competing compounds are present (e.g., LDL, asialoglycoproteins), a single blood sample may be analyzed for the concentration of these competing compounds and the results used to calculate a flux (micromoles/minute) through the clearance pathways.

15 In order to demonstrate utility of the invention, a non-invasive fluorescence detection system in accordance with the present invention was employed to continuously monitor dye removal from the vasculature. Differentiation between normal and abnormal organ function in a rat model was demonstrated
20 for both liver and kidney. With reference to Figure 1, a fiber optic 10 transmitted light from source 12 to ear 14. A second fiber optic 16 positioned near the ear 14 transmitted the fluorescent light to a detector system 20. Two dyes were employed in this initial study. Indocyanine green is
25 exclusively cleared from the blood stream by the liver, and was excited *in vivo* with laser light at 780 nm. The

fluorescence signal was detected at 830 nm. A characteristic clearance curve of normal hepatic function was obtained. Upon ablation of a portion of the liver, the clearance curve was greatly extended as expected. FITC labelled, succinylated 5 poly-D-lysine was excited *in vivo* with laser light at 488 nm. The fluorescence signal was detected at 518 nm. A characteristic clearance curve of normal renal function was obtained. Upon ligation of both kidneys, the clearance curve remained elevated and constant, indicating little if any 10 clearance. See Figure 2.

With the schematic apparatus for non-invasive *in vivo* detection of fluorescence shown in Figure 1, for ICG fluorescence detection, a nominal 785 nm collimated solid state laser source was employed (LaserMax Inc. model # LAS-15 300-780-5). For FITC fluorescence detection, an argon ion laser (Coherent Innova model 90) tuned to the 488 nm line was used. Either laser source was directed into the end of a 3.2 mm inner diameter glass fiber optic bundle 10 (Oriel #77526). The other end of this laser delivery bundle was placed 20 approximately 2 cm from the rat ear 14 at an approximate 45° angle. A second similar fiber optic bundle 16 for use as the fluorescence detection conduit was placed approximately 1 cm from the ear 14 at a 30° angle.

The exit end of the detection fiber bundle 16 was 25 positioned at the focal length of a 20 mm focal length lens 18. The output light was thus collimated after exiting the

bundle and passing through the lens. A narrow band interference filter 20 (IF) was the next element in the optics train (CVI Laser Corporation), allowing light of the appropriate wavelength to pass on to the detector 20. For the 5 ICG fluorescence experiment, an 830 nm filter (10 nm FWHM) was used. For the FITC fluorescence experiment, a 518 nm filter (3 nm FWHM) was used.

The detector 20 was a small silicon photodiode (UDT model PIN-10D) connected to a transimpedance amplifier (UDT model 10 101C). A digital voltmeter 22 (DVM) monitors the output signal. A subsequent voltage amplifier 24 (Tektronix AM-502) boosts the signal if needed. The amplifier output is connected to a National Instruments BNC-2080 breakout board, which is interfaced to a National Instruments DAQ-700 data 15 acquisition board 26 (A/D). LabVIEW® data acquisition software in computer 28 collects the experimental raw data.

The current method contrasts with the prior art methods which used radiolabeled tracers. The present method eliminates concerns about radioactivity and allows concurrent 20 measurements of different parameters simply by rapid alteration of the excitation and emission wavelengths.

A variety of dyes and carriers can be used for the disclosed methods. Dyes which can be used include phenylxanthenes (e.g., fluorescein), phenothiazines, 25 phenoselenazines, cyanines, indocyanines and squaraines. Preferred carriers are physiologically acceptable polyanionic

- compounds which may be conjugated to the above dyes. Carriers which can be used include polyacrylic acid, polyaspartic acid, polyglutamic acid, polynucleotides, polyarginine, polyserine, polyornithine and polylysine. The following dye-carrier conjugates can be used as part of the present invention with those marked with an "*" being the preferred conjugates.
- Those preceded by RF are used to test renal function; those preceded by LF are used to test liver function; and those preceded by RF/LF are used to test renal and liver function.
- 10 RF Fluorescein-polyaspartic acid*
- RF Fluorescein-polyglutamic acid*
- RF Fluorescein-polyacrylic acid*
- RF Fluorescein-polynucleotides
- RF Fluorescein-polynitrophenylalanine
- 15 RF Fluorescein-polydinitrophenylalanine
- RF Fluorescein-polytrinitrophenylalanine
- RF Fluorescein-polysulfonylphenylalanine
- RF Fluorescein-polydisulfonylphenylalanine
- RF Fluorescein-polytrisulfonylphenylalanine
- 20 RF Fluorescein-polysuccinate
- RF Fluorescein-polymalonate
- RF Fluorescein-polyglutarate
- RF Fluorescein-polyglycolate
- LF Bis(hexanoic acid)indocyanine green-polyaspartic acid*
- 25 LF Bis(hexanoic acid)indocyanine green-polyglutamic acid*
- LF Bis(hexanoic acid)indocyanine green-polyacrylic acid*

- LF Bis(hexanoic acid)indocyanine green-polynucleotides
- LF Bis(hexanoic acid)indocyanine green-
- polynitrophenylalanine
- LF Bis(hexanoic acid)indocyanine green-
- 5 polydinitrophenylalanine
- LF Bis(hexanoic acid)indocyanine green-
- polytrinitrophenylalanine
- LF Bis(hexanoic acid)indocyanine green-
- polysulfonylphenylalanine
- 10 LF Bis(hexanoic acid)indocyanine green-
- polydisulfonylphenylalanine
- LF Bis(hexanoic acid)indocyanine green-
- polytrisulfonylphenylalanine
- LF Bis(hexanoic acid)indocyanine green-polysuccinate
- 15 LF Bis(hexanoic acid)indocyanine green-polymalonate
- LF Bis(hexanoic acid)indocyanine green-polyglutarate
- LF Bis(hexanoic acid)indocyanine green-polyglycolate
- RF/LF Bis(propanoic acid)indocyanine green-polyaspartic acid*
- RF/LF Bis(propanoic acid)indocyanine green-polyglutamic acid*
- 20 RF/LF Bis(propanoic acid)indocyanine green-polyacrylic acid*
- RF/LF Bis(propanoic acid)indocyanine green-polynucleotides
- RF/LF Bis(propanoic acid)indocyanine green-
- polynitrophenylalanine
- RF/LF Bis(propanoic acid)indocyanine green-polydinitrophenylalanine
- 25 RF/LF Bis(propanoic acid)indocyanine green-
- polytrinitrophenylalanine

- RF/LF Bis(propanoic acid)indocyanine green-
polysulfonylphenylalanine
- RF/LF Bis(propanoic acid)indocyanine green-
polydisulfonylphenylalanine
- 5 RF/LF Bis(propanoic acid)indocyaninegreen-
polytrisulfonylphenylalanine
- RF/LF Bis(propanoic acid)indocyanine green-polysuccinate
- RF/LF Bis(propanoic acid)indocyanine green-polymalonate
- RF/LF Bis(propanoic acid)indocyanine green-polyglutarate
- 10 10 RF/LF Bis(propanoic acid)indocyanine green-polyglycolate
- RF Bis(benzothiazole)squareaine-polyaspartic acid*
- RF Bis(benzothiazole)squareaine-polyglutamic acid*
- RF Bis(benzothiazole)squareaine-polyacrylic acid*
- RF Bis(benzothiazole)squareaine-polynucleotides
- 15 15 RF Bis(benzothiazole)squareaine-polynitrophenylalanine
- RF Bis(benzothiazole)squareaine-polydinitrophenylalanine
- RF Bis(benzothiazole)squareaine-polytrinitrophenylalanine
- RF Bis(benzothiazole)squareaine-polysulfonylphenylalanine
- RF Bis(benzothiazole)squareaine-polydisulfonylphenylalanine
- 20 20 RF Bis(benzothiazole)squareaine-polytrisulfonylphenylalanine
- RF Bis(benzothiazole)squareaine-polysuccinate
- RF Bis(benzothiazole)squareaine-polymalonate
- RF Bis(benzothiazole)squareaine-polyglutarate
- RF Bis(benzothiazole)squareaine-polyglycolate
- 25 25 RF Bis(trihydroxyphenyl)squareaine-polyaspartic acid*
- RF Bis(trihydroxyphenyl)squareaine-polyglutamic acid*
- RF Bis(trihydroxyphenyl)squareaine-polyacrylic acid*

- RF Bis(trihydroxyphenyl)squaraine-polynucleotides
 - RF Bis(trihydroxyphenyl)squaraine-polynitrophenylalanine
 - RF Bis(trihydroxyphenyl)squaraine-polydinitrophenylalanine
 - RF Bis(trihydroxyphenyl)squaraine-polytrinitrophenylalanine
 - 5 RF Bis(trihydroxyphenyl)squaraine-polysulfonylphenylalanine
 - RF Bis(trihydroxyphenyl)squaraine-
polydisulfonylphenylalanine
 - RF Bis(trihydroxyphenyl)squaraine-
polytrisulfonylphenylalanine
 - 10 RF Bis(trihydroxyphenyl)squaraine-polysuccinate
 - RF Bis(trihydroxyphenyl)squaraine-polymalonate
 - RF Bis(trihydroxyphenyl)squaraine-polyglutarate
 - RF Bis(trihydroxyphenyl)squaraine-polyglycolate
- The invention is further illustrated by the following
15 examples, which are not intended to be limiting.

Example 1

For these in-vivo studies, normal Sprague-Dawley rats weighing ~250 grams were first anesthetized with urethane (1.35 g/kg) administered via intraperitoneal injection.

5 After each animal had achieved the desired plane of anesthesia, a small (0.5 cm) incision was made in the upper thorax exposing the left jugular vein. The lobe of the left ear was fixed to a glass microscope slide, and the incident laser light delivered from the fiber optic was centered on
10 that ear. Data acquisition was then initiated, and a background reading of fluorescence was obtained prior to administration of the test article. Next, the dye (ICG for liver clearance assessment, FITC labeled poly-D-lysine for kidney clearance assessment) was administered via the jugular
15 vein. The fluorescence signal immediately increased to a peak value. The signal decayed as a function of time as the dye presumably cleared from the bloodstream.

The anesthetized rat was placed on its back and a midline ventral abdominal skin incision made extending from the
20 xiphoid cartilage to approximately midway to the tail. A similar incision was then made in the abdominal muscles exposing the liver. The rat was repositioned and a bolster placed under the thorax to cause the liver to fall slightly forward and away from the diaphragm. The median and left
25 lateral lobes of the liver were gently moved out of the abdominal cavity and placed onto a gauze pad wetted with

saline. The two lobes were vertically raised and a 3-0 ligature placed around the isolated lobes but were not ligated at this point of the procedure. The lobes were replaced in the abdominal cavity and the bolster removed. The incision 5 was closed with wound clips.

ICG was administered to the animal via the exposed jugular vein as in the previous study. The clearance of the test article was monitored as before to determine the normal hepatic clearance of the ICG. After the normal clearance 10 curve was obtained, the ligature around the two isolated lobes of the liver was tied securely to effect a partial hepatectomy. The animal was allowed to equilibrate for 20 minutes in this state. The ICG was next administered via the exposed jugular vein, and the clearance of the test article 15 monitored. Clearance curves of normal versus partial hepatectomized animals were obtained for an n=3 sample.

The time dependence of fluorescence measured at the ear pre and post bolus injection of ICG for three rats is shown in Figure 3. The data can be described in terms of three stages. 20 Stage 1 consisted of approximately the first 30 seconds of data, which was gathered pre-bolus injection. These data were constant and represented the baseline value for the forthcoming experiment. The value of the baseline should be zero, since no fluorescence is occurring during this stage. 25 Stage 2 occurred several seconds post-injection, the signal rapidly rose to a maximum as the dye reached the ear and

equilibrated in the blood pool. In the third stage, the fluorescence signal decayed with time as the liver filtered the ICG out of the blood stream. Visually, the decay rates were similar for all three. After 15 minutes, approximately 5 90% of the initial signal was lost.

To verify that the measurement was indeed that of ICG fluorescence, the following control study was performed. A rat was injected, as above, with 500 μ L of 1.41 mM ICG. A normal fluorescence time course was obtained and is labeled as 10 ICG-1 in Figure 4. Then the same rat was injected with 500 μ L of 1.41 mM fluorescein (Sigma, St. Louis, MO). As shown in Figure 4, no fluorescence signal was detected. As a further check, 500 μ L of saline solution (Baxter, Deerfield, IL) was injected into the same rat next. Again, no detectable signal 15 was obtained. Finally, the rat was once again injected with 500 μ L of 1.41 mM ICG, and a second "normal" curve was obtained.

To verify that these fluorescence decay curves were related to liver function, an experiment involving a partial 20 liver ablation was performed. The partial liver ablation procedure is outlined above. Once the surgery was complete, and the ligatures for use in partially ablating the liver were ready, the rat was injected with 500 μ L of 1 mM ICG solution. A normal fluorescence time course curve was obtained and is 25 shown in Figure 5.

The liver was then partially ablated by tightening the ligatures. The rat was allowed to equilibrate for ten minutes. Next, another injection of 500 μ L of 1 mM ICG was given. The fluorescence time curve was measured and is also shown in Figure 5. The capability of the liver to remove ICG from the blood pool was drastically altered, the fluorescence decay rate for the partially ablated liver was much slower than the normal. Upon sacrifice, the liver was weighed and 44% of the liver was found to be ablated.

10

Example 2

500 mg (~125 mole equivalent lysine) poly-D-lysine 4000 (Sigma P-0296) was dissolved in 10 mL 0.1 M Na_2CO_3 , in a dark glass vial with magnetic stirring bar. 24.3 mg (62.5 μ moles) FITC (Fluorescein isothiocyanate, Sigma F-7250) was dissolved 15 in 1.5 mL DMSO (dimethyl sulfoxide). At 25°C, with stirring, the FITC solution was slowly added to the poly-D-lysine solution. The reaction was allowed to proceed for 30 minutes at 25°C, then transferred to 4°C, and stirred 12 hrs. The fluorescein conjugate was separated from unbound FITC by gel 20 filtration on Sephadex G-25, eluting with 0.9% (w/v) NaCl.

20 mL 0.9% NaCl containing ~30 μ moles of the above conjugate were placed in a dark glass vial with magnetic stirring bar and pH electrode at 25°C; pH was raised to 9.5 by addition of 0.5 M NaOH. 500 mg of succinic anhydride was 25 added slowly, with stirring, to this solution over a period of 30 minutes, maintaining pH 9.5-10.0 by the addition of 0.5 M

NaOH. The pH was then allowed to fall to a stable value of 7.5, with a final volume of 27 mL. The reaction mixture was dialyzed vs. 0.9% (w/v) NaCl using a dialysis membrane with 3.5 kd cutoff, and the retained polymer conjugate, at ~4.0 μ M 5 fluorescein concentration equivalent, was used directly for infusion.

The anesthetized rat was placed on the ventral surface and bilateral dorsoventral incisions were made in the abdominal cavity near to the costal border of the thorax. 10 The kidneys were freed of connective tissue and were gently pulled away from the abdomen by grasping the perirenal fat tissue. A single 3-0 ligature was placed around the renal vessels and ureter so as not to occlude collateral vessels. The ligatures were not tied at this point of the procedure.

15 Succinylated, fluorescein-labeled poly-D-lysine was administered via the exposed jugular vein. The clearance of the test article was monitored as before to determine the normal renal clearance of poly-D-lysine. After the normal clearance curve was obtained, the ligature was tied to effect 20 a total (bilateral) nephrectomy. The animal was allowed to stabilize in this condition for 20 minutes. The test article was next administered via the exposed jugular vein and the clearance of the compound monitored. Clearance curves of normal versus total nephrectomized animals were obtained for 25 an n=3 sample.

Example 3

Preparation of fluorescein-polyaspartic acid (6000) conjugate

A solution of fluorescein isothiocyanate (20 mg) in DMSO (0.5 mL) was added to a solution of polyaspartic acid (MW: 6000, 180 mg) in water (1 mL). The reddish-orange homogeneous solution was kept at ambient temperature for 24 hours. The reaction mixture was treated with acetone (20 mL). The fine precipitate was separated by centrifugation. The supernatant was discarded and the precipitate resuspended in acetone (10 mL). This washing process was repeated three more times to remove bulk of unreacted fluorescein isothiocyanate. The pellet was dissolved in water (0.5 mL) and chromatographed through Sephadex G-25 (prefilled Pharmacia PD-10) column. One milliliter fractions were collected. The desired fractions (1-4) were pooled and lyophilized to give 87 mg of the desired conjugate as an orange solid. Absorbance of 0.21 mg/mL solution was 0.26, which corresponds to 10.2% conjugation. The procedure of Example 1 was followed using an excitation wavelength of 488 nanometers and an emission wavelength of 520 nanometers. Figure 6 shows the blood clearance profile of fluorescein-polyaspartic acid (6000) conjugate.

Example 4Preparation of fluorescein-polyaspartic acid (10000) conjugate

A solution of fluorescein isothiocyanate (20 mg) in DMSO (0.5 mL) was added to a solution of polyaspartic acid (MW: 10000, 180 mg) in water (1 mL). The reddish-orange

homogeneous solution was kept at ambient temperature for 24 hours. The reaction mixture was treated with acetone (20 mL). The fine precipitate was separated by centrifugation. The supernatant was discarded and the precipitate resuspended in 5 acetone (10 mL). This washing process was repeated three more times to remove bulk of unreacted fluorescein isothiocyanate. The pellet was dissolved in water (0.5 mL) and chromatographed through Sephadex G-25 (prefilled Pharmacia PD-10) column. The desired fractions (1-4) were pooled and lyophilized to give 10 100 mg of the desired conjugate as an orange solid. Absorbance of 0.19 mg/mL solution was 0.05, which corresponds to 3.3% conjugation. The procedure of Example 1 was followed using an excitation wavelength of 488 nanometers and an emission wavelength of 520 nanometers. Figure 7 shows the 15 blood clearance profile of fluorescein-polyaspartic acid (10000) conjugate.

Example 5

Preparation of fluorescein-polyglutamic acid (13000) conjugate

A solution of fluorescein isothiocyanate (20 mg) in DMSO 20 (0.5 mL) was added to a solution of polyglutamic acid (MW: 13000, 150 mg) in water (1 mL). The reddish-orange homogeneous solution was kept at ambient temperature for 24 hours. The reaction mixture was treated with acetone (20 mL). The fine precipitate was separated by centrifugation. The 25 supernatant was discarded and the precipitate resuspended in acetone (10 mL). This washing process was repeated three more

times to remove bulk of unreacted fluorescein isothiocyanate. The pellet was dissolved in water (0.5 mL) and chromatographed through Sephadex G-25 (prefilled Pharmacia PD-10) column. The desired fractions (1-4) were pooled and lyophilized to give 80 mg of the desired conjugate as an orange solid. Absorbance of 0.21 mg/mL solution was 0.79, which corresponds to 60.8% conjugation. The procedure of Example 1 was followed using an excitation wavelength of 488 nanometers and an emission wavelength of 520 nanometers. Figure 8 shows the blood 10 clearance profile of fluorescein-polyglutamic acid (13000) conjugate.

Example 6

Preparation of fluorescein-polyarginine (10000) conjugate

A solution of fluorescein isothiocyanate (20 mg) in DMSO 15 (0.5 mL) was added to a solution of polyarginine (MW: 10000, 150 mg) in water (1 mL). The reddish-orange homogeneous solution was kept at ambient temperature for 24 hours. The reaction mixture was treated with acetone (20 mL). The fine precipitate was separated by centrifugation. The supernatant 20 was discarded and the precipitate resuspended in acetone (10 mL). This washing process was repeated three more times to remove bulk of unreacted fluorescein isothiocyanate. The pellet was dissolved in water (0.5 mL) and chromatographed through Sephadex G-25 (prefilled Pharmacia PD-10) column. The 25 desired fractions (1-4) were pooled and lyophilized to give 80 mg of the desired conjugate as an orange solid. Absorbance of

0.21 mg/mL solution was 0.73, which corresponds to 60.5% conjugation. The procedure of Example 1 was followed using an excitation wavelength of 488 nanometers and an emission wavelength of 520 nanometers. Figure 9 shows the blood clearance profile of fluorescein-polyarginine (10000) conjugate.

Example 7

Preparation of indocyanine-dodecaaspartic acid conjugate

(a) Peptide synthesis: Dodecaaspartic acid mono and bis aspartimide were prepared by solid phase Fmoc peptide synthesis with automated Applied Biosystems 432A Synergy synthesizer. The amino acid units (75 micromoles each) were sequentially activated with a mixture of N-hydroxybenzotriazole (HOBT) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The Fmoc of the terminal amino acid was removed with 20% piperidine in dimethylformamide.

(b) Dye synthesis: The near infrared absorbing bis(carboxyhexyl)indocyanine dye (NIRD) was prepared by the reaction of 1,1,2-trimethyl-[1H]-benz[e]indole (20 g, 95.6 mmoles) and 6-bromohexanoic acid (28.1 g, 144.1 mmoles) in 1,2-dichlorobenzene (DCB) at 110°C for 12 hours. The green solution was cooled to room temperature and the brown solid precipitate formed was collected by filtration. After washing the solid with DCB and diethyl ether, the brown powder obtained (24 g, 64%) was dried under vacuum at room

temperature. A portion of this solid (4.0 g, 9.8 mmoles), glutaconaldehyde dianil monohydrochloride (1.4 g, 5 mmoles) and sodium acetate trihydrate (1.8 g, 12.9 mmoles) were refluxed in ethanol for 1 hour. After evaporating the solvent, 20 mL of a 2N aqueous HCl solution was added to the residue and the mixture was centrifuged and the supernatant was decanted. This procedure was repeated until the supernatant became nearly colorless. About 5 mL of a mixture of water:acetonitrile (3:2) was added to the solid residue which was lyophilized to obtain about 2 g of dark green flakes. Purity of the compound was established with ¹H-NMR and LC-mass spectrometry.

(c) Dye-peptide conjugate: The dye (60 mg, 75 micromoles) was added to an activation reagent consisting of 0.4 mL HBTU in DMSO (0.2 M) and 0.4 mL diisopropylethylamine in DMSO (0.4 M). The activation was complete in about 30 minutes and the resin-bound peptide (25 micromoles) was added to the dye. The reaction was carried out at room temperature for 3 hours. The mixture was filtered and the solid residue was washed with DMG, acetonitrile and THF. After drying the green residue, the aspartic acid side chain protecting group and peptide cleavage from the resin were accomplished in one step with a mixture of 85% trifluoroacetic acid, 5% water, 5% thioanisole and 5% phenol. The resin was filtered and cold t-butyl methyl ether (MTBE) was used to precipitate the dye-peptide conjugate which was lyophilized and purified by HPLC.

Two compounds, mono- and bis-aspartimides of dodecaaspartic acid, were obtained.

The procedure of Example 1 was followed using an excitation wavelength of 780 nanometers and an emission wavelength of 830 nanometers. Figure 10 shows the blood clearance profile of indocyanine-mono(polyaspartic acid 6000).

Example 8

Preparation of indocyanine

(NIRD)-polyaspartic acid 6000 conjugate

10 NIRD (60 mg, 75 micromoles) was activated with a mixture of 0.4 mL HBTU in DMSO (0.2 M) and 0.22 mL diisopropylethylamine in DMSO (0.4 M) for 1 hour.

Polyaspartic acid (6 kDa, 100 mg) was added to the dye and the conjugation was complete after 3 hours. Cold MTBE was added

15 (4 x 10 mL) to precipitate the peptide conjugate and wash off reaction solvents and reagents. The green paste obtained was dissolved in water and lyophilized to give a green solid. The compound was purified by gel filtration chromatograph with Pharmacia PD-10 column (Sephadex G-25) by eluting with PBS.

20 The product was lyophilized and analyzed by HPLC, UV-visible spectrophotometry, fluorometry and mass spectrometry. The procedure of Example 1 was followed using an excitation wavelength of 780 nanometers and an emission wavelength of 830 nanometers. Figure 11 shows the blood clearance profile of

25 indocyanine (polyaspartic acid 6000).

The utility of non-invasive fluorescence detection to monitor liver or kidney function has been established.

The steps of the invention may be repeated in order to determine if physiological function is changing.

5 Indocyanine green is a dye which fluoresces at a wavelength of about 830 nm and was used to measure the physiological function of the liver. In order to measure the physiological function of the liver, a body portion was irradiated with light with a wavelength of about 780 nm. The 10 physiological or hepatic function of liver cells was measured using the claimed method.

Fluorescein labeled, succinylated poly-D-lysine is a dye which fluoresces at a wavelength of about 518 nm and was used to measure the physiological function of the kidneys. In 15 order to measure the physiological function of the kidneys, a body portion was irradiated with light with a wavelength of about 488 nm. Renal function was measured using the above-described method of the invention. See Figure 2.

The dyes were intravenously injected. A body portion, 20 which included blood vessels near the surface of the skin, was irradiated with a laser or with infrared radiation.

The claimed invention may also be used to evaluate hypercholesterolemia. Clearance rate measurements may allow the clinician to determine whether high serum cholesterol 25 resulted from increased rate of LDL production or from decreased rate of LDL clearance, which may impact therapy.

The claimed invention may also be used to measure cardiac output. The ability to concurrently measure cardiac function while also measuring hepatic and renal function may allow the clinician to draw preliminary conclusions about whether any 5 observed changes in hepatic and renal functions were due to primary renal or hepatic disease or secondary to heart disease.

Since many modifications, variations and changes in detail may be made to the described embodiments, it is 10 intended that all matter in the foregoing description and shown in the accompanying drawings be interpreted as illustrative and not in a limiting sense.

CLAIMS

- 1 1. A method of measuring physiological function of a
2 group of cells in a patient's body selected from the group
3 consisting of renal or hepatic cells comprising the steps of:
4 a) selecting a detectable agent which emits an
5 electromagnetic emission, said agent being selectively removed
6 from a body fluid in said patient's body by said group of
7 cells in said patient's body, and wherein said emission occurs
8 in said body fluid in said patient's body;
9 b) introducing said agent into said body fluid of a
10 patient, which body fluid contacts said group of cells in said
11 patient's body, and wherein said emission occurs in said body
12 fluid;
13 c) measuring said emission from a body portion in said
14 patient's body through which said body fluid passes wherein
15 said emission results from agent not yet removed from said
16 body fluid at a time of said measuring; and
17 d) determining said physiological function based on
18 measurement of said emission,
19 wherein said agent includes a dye which fluoresces
20 at a first wavelength upon being irradiated with light of
21 a second wavelength and wherein said dye is conjugated to
22 a physiologically acceptable polyanionic carrier;
23 wherein prior to said measuring step, said body
24 portion through which said body fluid passes is

25 irradiated with said light of said second wavelength,
26 causing said dye to fluoresce at said first wavelength;
27 wherein said emission being measured is fluorescence
28 of said dye at said first wavelength; and
29 wherein said determination of physiological function
30 is based on measurement of fluorescence of said first
31 wavelength.

1 . 2. The method of claim 1 wherein said dye is selected
2 from the group consisting of phenylxanthenes, phenothiazines,
3 phenoselenazines, cyanines, indocyanines and squaraines.

1 3. The method of claim 1 wherein said physiologically
2 acceptable polyanionic carrier is selected from the group
3 consisting of polyacrylic acid, polyaspartic acid,
4 polyglutamic acid, polynucleotides, polyarginine, polyserine,
5 polyornithine and polylysine.

1 4. The method of claim 1, wherein said agent is a dye
2 selected from the group consisting of:
3 Fluorescein-polyaspartic acid,
4 Fluorescein-polyglutamic acid,
5 Fluorescein-polyacrylic acid,
6 Fluorescein-polynucleotides,
7 Fluorescein-polynitrophenylalanine,
8 Fluorescein-polydinitrophenylalanine,

9 Fluorescein-polytrinitrophenylalanine,
10 Fluorescein-polysulfonylphenylalanine,
11 Fluorescein-polydisulfonylphenylalanine,
12 Fluorescein-polytrisulfonylphenylalanine,
13 Fluorescein-polysuccinate,
14 Fluorescein-polymalonate,
15 Fluorescein-polyglutarate,
16 Fluorescein-polyglycolate,
17 Bis(hexanoic acid)indocyanine green-polyaspartic acid,
18 Bis(hexanoic acid)indocyanine green-polyglutamic acid,
19 Bis(hexanoic acid)indocyanine green-polyacrylic acid,
20 Bis(hexanoic acid)indocyanine green-polynucleotides,
21 Bis(hexanoic acid)indocyanine green-
22 polynitrophenylalanine,
23 Bis(hexanoic acid)indocyanine green-
24 polydinitrophenylalanine,
25 Bis(hexanoic acid)indocyanine green-
26 polytrinitrophenylalanine,
27 Bis(hexanoic acid)indocyanine green-
28 polysulfonylphenylalanine,
29 Bis(hexanoic acid)indocyanine green-
30 polydisulfonylphenylalanine,
31 Bis(hexanoic acid)indocyanine green-
32 polytrisulfonylphenylalanine,
33 Bis(hexanoic acid)indocyanine green-polysuccinate,
34 Bis(hexanoic acid)indocyanine green-polymalonate,

35 Bis(hexanoic acid)indocyanine green-polyglutarate,
36 Bis(hexanoic acid)indocyanine green-polyglycolate,
37 Bis(propanoic acid)indocyanine green-polyaspartic acid,
38 Bis(propanoic acid)indocyanine green-polyglutamic acid,
39 Bis(propanoic acid)indocyanine green-polyacrylic acid,
40 Bis(propanoic acid)indocyanine green-polynucleotides,
41 Bis(propanoic acid)indocyanine green-
42 polynitrophenylalanine,
43 Bis(propanoic acid)indocyanine green-polydinitrophenylalanine,
44 Bis(propanoic acid)indocyanine green-
45 polytrinitrophenylalanine,
46 Bis(propanoic acid)indocyanine green-
47 polysulfonylphenylalanine,
48 Bis(propanoic acid)indocyanine green-
49 polydisulfonylphenylalanine,
50 Bis(propanoic acid)indocyaninegreen-
51 polytrisulfonylphenylalanine,
52 Bis(propanoic acid)indocyanine green-polysuccinate,
53 Bis(propanoic acid)indocyanine green-polymalonate,
54 Bis(propanoic acid)indocyanine green-polyglutarate,
55 Bis(propanoic acid)indocyanine green-polyglycolate,
56 Bis(benzothiazole)squareaine-polyaspartic acid,
57 Bis(benzothiazole)squareaine-polyglutamic acid,
58 Bis(benzothiazole)squareaine-polyacrylic acid,
59 Bis(benzothiazole)squareaine-polynucleotides,
60 Bis(benzothiazole)squareaine-polynitrophenylalanine,

61 Bis(benzothiazole)squaraine-polydinitrophenylalanine,
62 Bis(benzothiazole)squaraine-polytrinitrophenylalanine,
63 Bis(benzothiazole)squaraine-polysulfonylphenylalanine,
64 Bis(benzothiazole)squaraine-polydisulfonylphenylalanine,
65 Bis(benzothiazole)squaraine-polytrisulfonylphenylalanine,
66 Bis(benzothiazole)squaraine-polysuccinate,
67 Bis(benzothiazole)squaraine-polymalonate,
68 Bis(benzothiazole)squaraine-polyglutarate,
69 Bis(benzothiazole)squaraine-polyglycolate,
70 Bis(trihydroxyphenyl)squaraine-polyaspartic acid,
71 Bis(trihydroxyphenyl)squaraine-polyglutamic acid,
72 Bis(trihydroxyphenyl)squaraine-polyacrylic acid,
73 Bis(trihydroxyphenyl)squaraine-polynucleotides,
74 Bis(trihydroxyphenyl)squaraine-polynitrophenylalanine,
75 Bis(trihydroxyphenyl)squaraine-polydinitrophenylalanine,
76 Bis(trihydroxyphenyl)squaraine-polytrinitrophenylalanine,
77 Bis(trihydroxyphenyl)squaraine-polysulfonylphenylalanine,
78 Bis(trihydroxyphenyl)squaraine-
79 polydisulfonylphenylalanine,
80 Bis(trihydroxyphenyl)squaraine-
81 polytrisulfonylphenylalanine,
82 Bis(trihydroxyphenyl)squaraine-polysuccinate,
83 Bis(trihydroxyphenyl)squaraine-polymalonate,
84 Bis(trihydroxyphenyl)squaraine-polyglutarate, and
85 Bis(trihydroxyphenyl)squaraine-polyglycolate.

1 5. A method as defined by claim 1, wherein steps b)
2 through d) are repeated to determine if physiological function
3 changes.

1 6. A method as defined by claim 1, wherein said agent
2 is injected.

1 7. A method as defined by claim 1, wherein said agent
2 is intravenously injected.

1 8. A method as defined by claim 1, wherein said body
2 portion includes blood vessels near a surface of skin of said
3 patient.

1 9. A method as defined by claim 1, wherein said second
2 wavelength is about 400 - 1200 nanometers.

1 10. A method as defined by claim 1, wherein said second
2 wavelength is about 488 nanometers.

1 11. A method as defined by claim 1, wherein said body
2 portion is irradiated with a laser.

1 12. A method as defined by claim 1, wherein said body
2 portion is irradiated with infrared radiation.

1 13. A method as defined by claim 1, wherein said body
2 cells are kidney cells.

1 14. A method as defined by claim 1, wherein said body
2 cells are liver cells.

1 15. A method as defined by claim 1, wherein said first
2 wavelength is about 830 nanometers.

1 16. A method as defined in claim 1, wherein said first
2 wavelength is about 518 nanometers.

1 17. A method as defined by claim 1, wherein said
2 physiological function is renal function.

1 18. A method as defined by claim 1, wherein said
2 physiological function is hepatic function.

1 19. A method as defined by claim 1, wherein said
2 emission is measured invasively or non-invasively with respect
3 to said body portion.

1 20. A method as defined by claim 19, wherein said
2 emission is endoscopically measured.

1 21. A method as defined by claim 19, further including
2 the step of inserting a catheter into said body portion so as
3 to measure said emission.

1 22. A method as defined by claim 19, wherein said body
2 portion is an ear of the patient's body.

1 23. A method as defined by claim 19, wherein said body
2 portion is a finger of the patient's body.

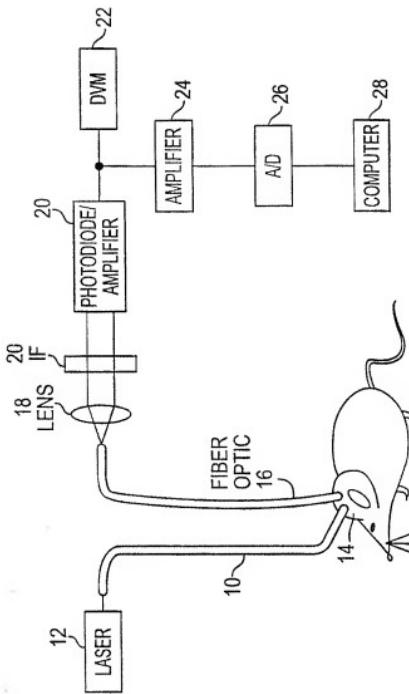


FIG. 1

2/11

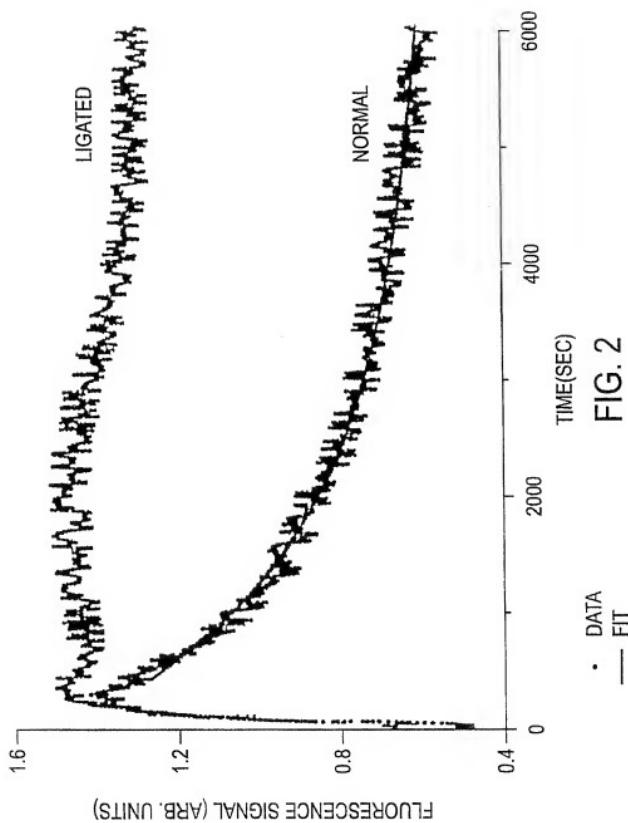


FIG. 2

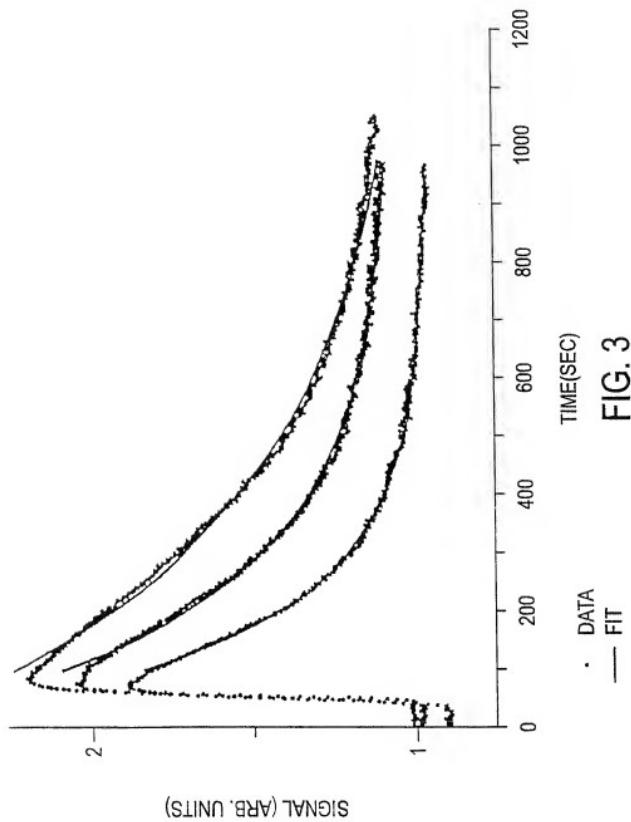


FIG. 3

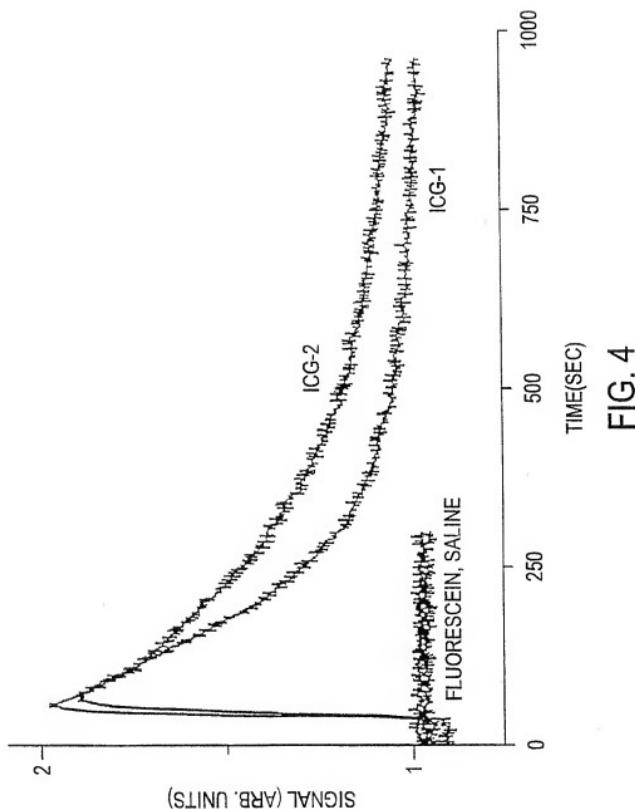
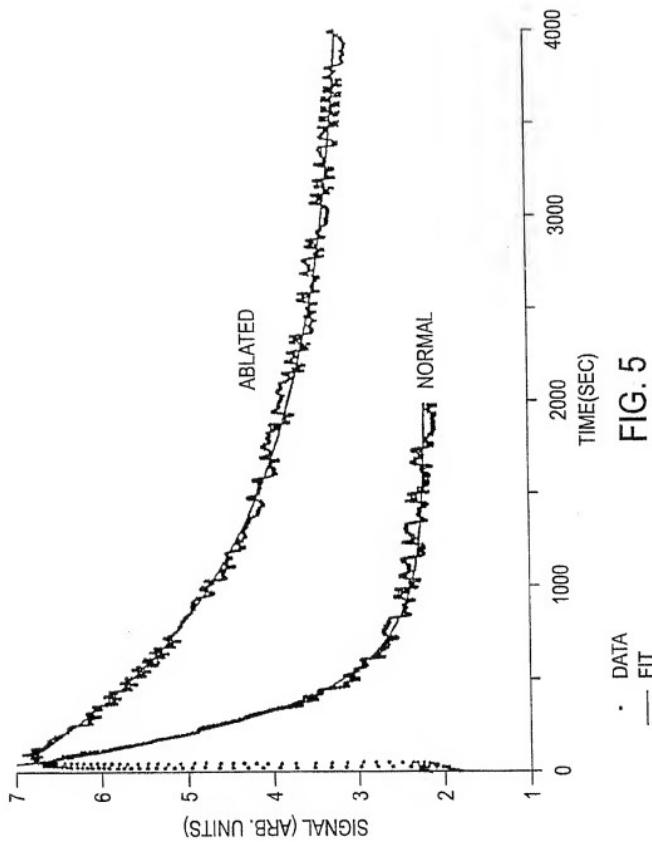
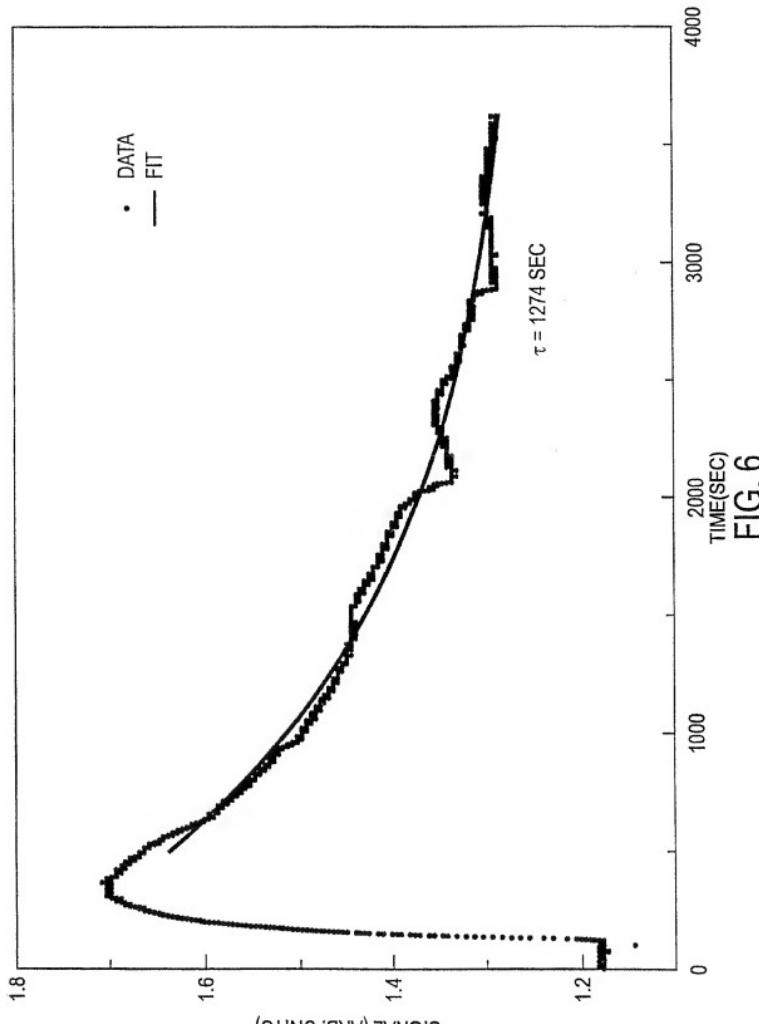


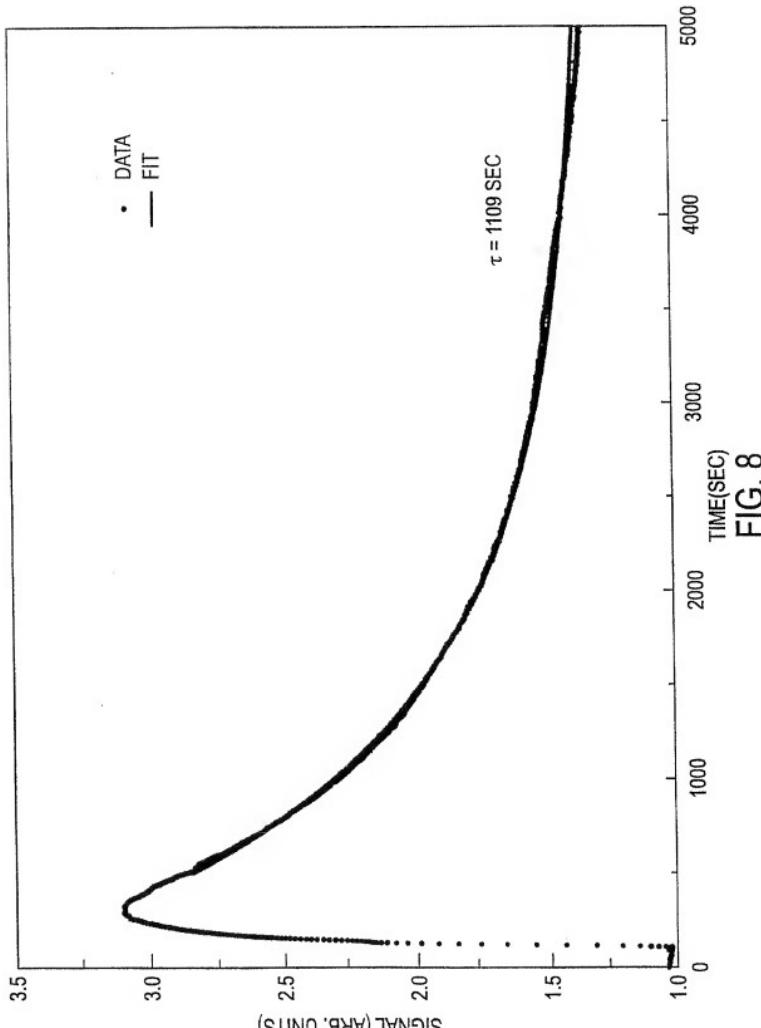
FIG. 4



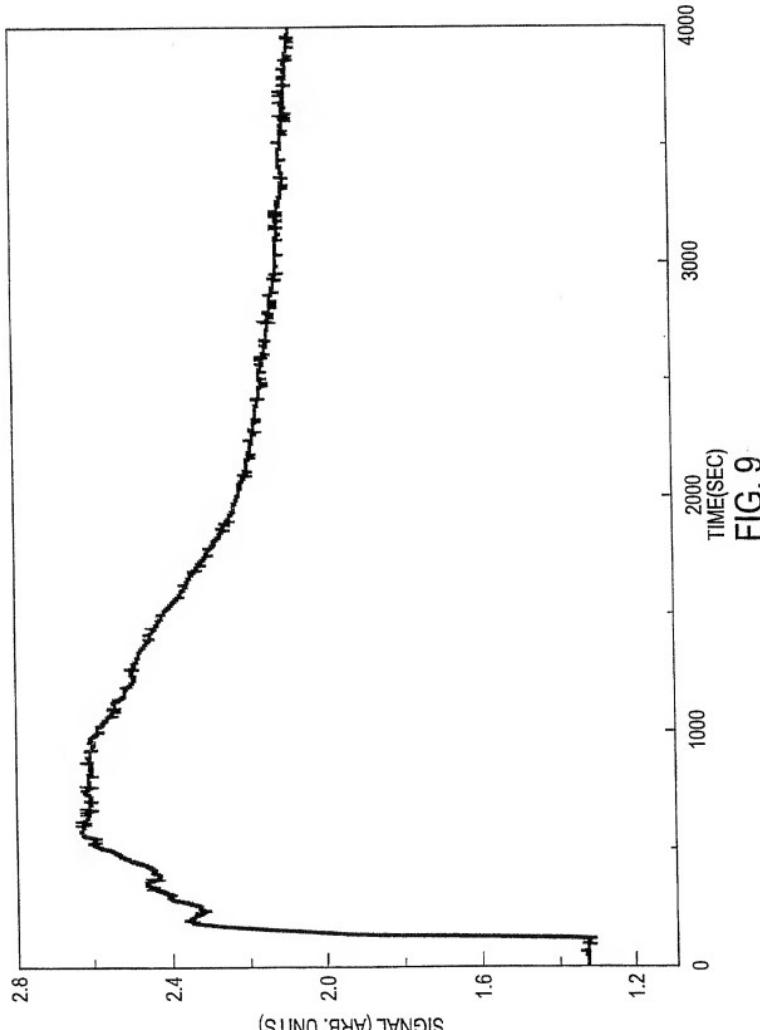
6/11



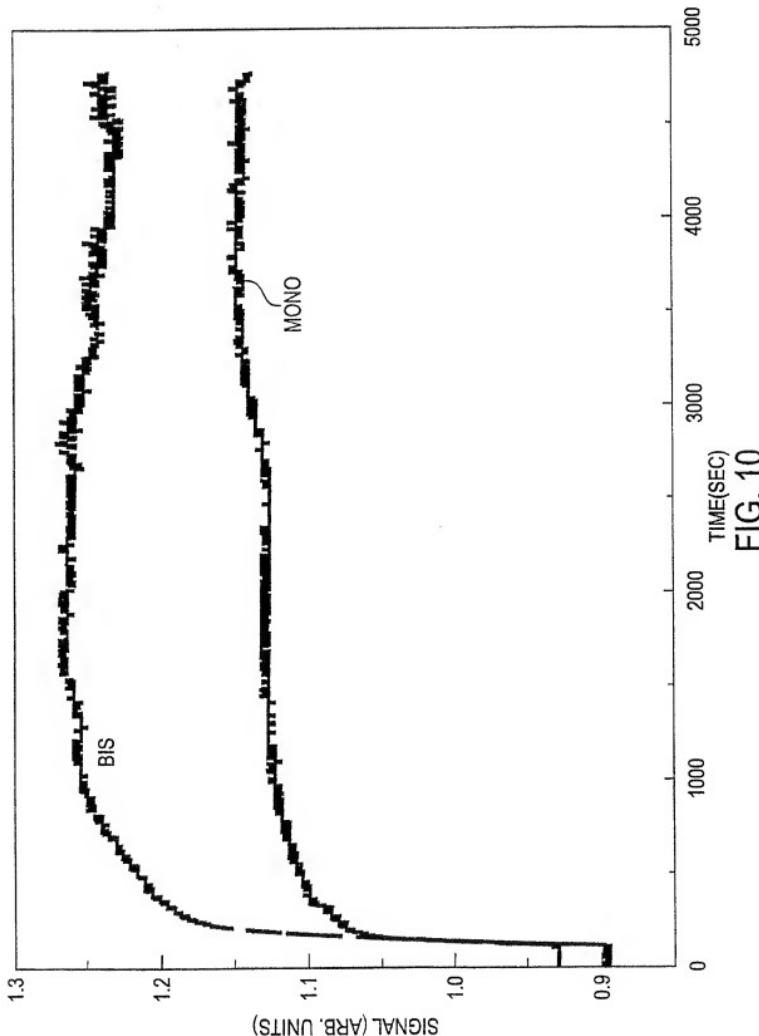
8/11



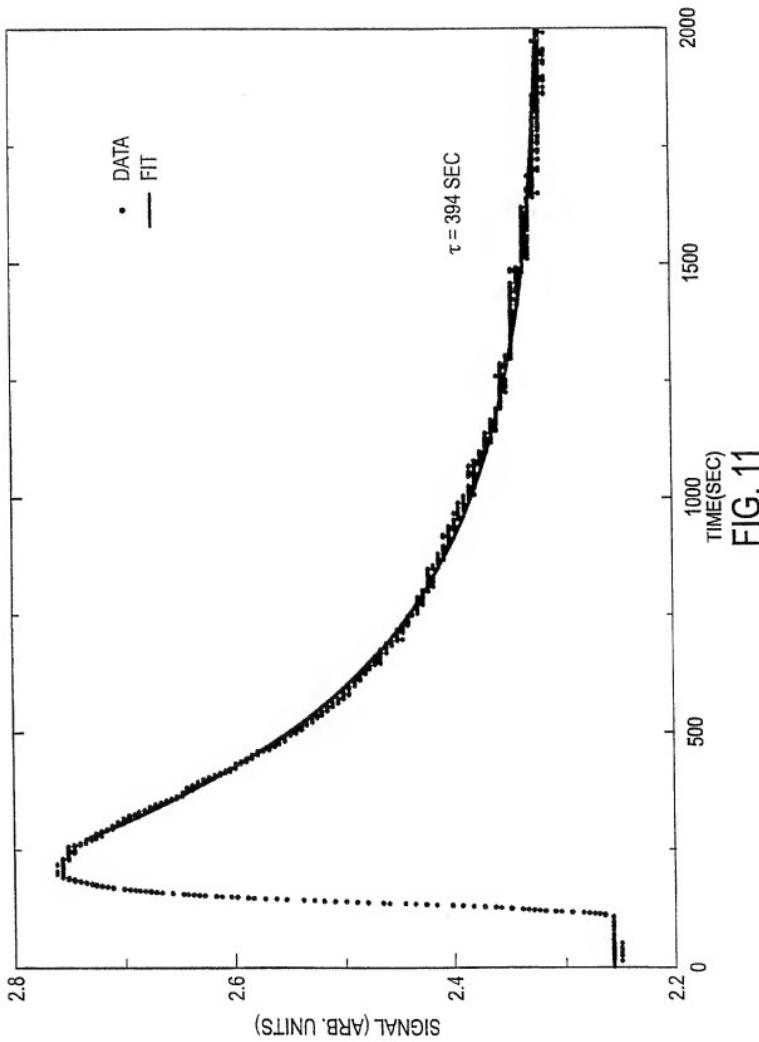
9/11



10/11



11/11



INTERNATIONAL SEARCH REPORT

Int'l. Appl. No.
PCT/US 00/01322A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 40106 A (UNIV WASHINGTON ;MALLINCKRODT MEDICAL INC (US)) 17 September 1998 (1998-09-17) claims 1,8,11	1-23
X		1
A	SOULI S ET AL: "IN VIVO PHARMACOKINETIC STUDY OF TWO FLUORESCIN DERIVATIVES BY FLUORESCENCE SPECTROSCOPY" PROCEEDINGS OF THE SPIE, US, SPIE, BELLINGHAM, VA, vol. 2627, 14 September 1995 (1995-09-14), pages 109-117, XP002071643	1-23

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

17 April 2000

Date of mailing of the International search report

02/05/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5018 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-9015

Authorized officer

Berte, M

INTERNATIONAL SEARCH REPORT

Int. oral Application No
PCT/US 00/01322

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE CHEMABS 'Online' CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US URATA, KOICHI ET AL: "Clinical evaluation of indocyanine green clearance using the finger-piece method in patients undergoing hepatic surgery" retrieved from STN Database accession no. 118:78470 XP002135872 abstract & YAKURI TO CHIRYO (1992), 20(SUPPL. 10), S2551-S2556 , ----- DE 44 45 065 A (DIAGNOSTIKFORSCHUNG INST) 13 June 1996 (1996-06-13) claims -----	1-23
Y		1-23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 00/01322

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-23 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of Invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. /oral Application No PCT/US 00/01322
--

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9840106	A	17-09-1998	US	5928625 A	27-07-1999
			AU	6046998 A	29-09-1998
			EP	0966306 A	29-12-1999
DE 4445065	A	13-06-1996	AU	709152 B	19-08-1999
			AU	3740995 A	26-06-1996
			CA	2205906 A	13-06-1996
			CN	1174511 A	25-02-1998
			WO	9617628 A	13-06-1996
			EP	0796111 A	24-09-1997
			HU	77378 A	28-04-1998
			JP	10510250 T	06-10-1998
			NO	972509 A	02-06-1997
			NZ	294568 A	30-08-1999
			ZA	9509707 A	29-05-1996

7/11

